Drug Delivery Systems Based on Trimethyl Lock Lactonization: Poly(ethylene glycol) Prodrugs of Amino-Containing Compounds

Richard B. Greenwald,* Yun H. Choe, Charles D. Conover, Kwok Shum, Dechun Wu, and Maksim Royzen

Enzon, Inc., 20 Kingsbridge Road, Piscataway, New Jersey 08854

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A novel methodology for the synthesis of poly(ethylene glycol) (PEG) prodrugs of amino-containing compounds has been developed which is based on the trimethyl lock lactonization reaction. These PEG-modified double prodrugs are water soluble, and by selective modification of the specifier or trigger, plasma half-lives can be adjusted at will to result in a wide range of values. Facile syntheses of ester, carbonate, and carbamate functionalities were accomplished and combined with greater or lesser degrees of steric hindrance in the spacer group, or on the aromatic framework, to achieve predictable ranges of drug concentration in plasma. In vivo screening of PEG prodrugs was done using a M109 syngeneic solid mouse tumor model. One of the PEG−daunorubicin prodrugs, with a half-life of 2 h, was evaluated in an in vivo solid tumor panel and found to be more efficacious against ovarian tumors (SKOV3) than equivalent amounts of daunorubicin.

Introduction

The lactonization of *o*-hydroxyphenylpropionic acid derivatives was first investigated in depth by Cohen[1] who defined the kinetic and structural parameters for the ring-closure reaction (usually referred to as the trimethyl lock (TML) effect). Subsequently, other groups further refined the kinetic details of the lactonization.[2] These findings ultimately formed the basis for an important class of amino protecting groups and prodrugs based on the β,β-dimethylpropionic acid amide side chain of the aromatic system. To effectively utilize these reactive compounds as prodrugs, the *ortho* phenolic group, similarly to 1,4- and 1,6-elimination-based prodrugs,[3] must necessarily first be modified to stabilize the reactive system. This is accomplished by synthesizing acyl phenolic derivatives that can be predictably hydrolyzed, or through a (bio)reductive mechanism which generates a phenol, in essence, by forming a double prodrug,[4] or tripartate system,[5] which consists of a trigger[6] (or specifier),[5] a linker, and the drug (amino or hydroxyl compound) to be released. Thus, regeneration of the OH group becomes the rate-determining step at which amine (drug) is generated (Figure 1). This process has been referred to as an esterase-mediated amide hydrolysis.[7]

The application of the chemistry of this intriguing cyclization reaction was initially investigated by Carpino,[8] utilizing a reductive approach to the TML system with simple amine models. Carpino first suggested in this study that prodrugs of this sort would have potential applicability in treating solid tumors. Subsequent efforts have been reported which also utilize a reductive activation trigger.[9] However, the TML double-prodrug approach employing esterase-sensitive specifiers to generate amines has been extensively developed by Borchardt and co-workers.[7,10] By altering the enzyme-sensitive specifier to an amino acid ester, extension of the methodology has led to the release of a model hexapeptide.[11] Most recently, Wang[12] has shown that cyclization (lactonization) of *o*-hydroxy-*cis*-cinnamic acid amides to coumarins, rapidly releasing amines, also lends itself to the list of feasible lactonization methods to consider for prodrug strategies. Prodrug strategies based on the TML approach as well as other intramolecular cyclization reactions have recently been reviewed.[13]

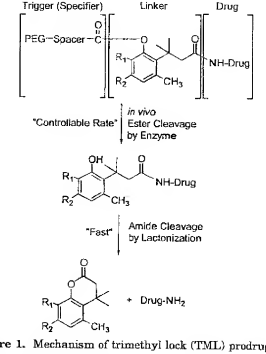

Figure 1. Mechanism of trimethyl lock (TML) prodrugs.

The use of the TML double-prodrug approach has also been adapted to release alcohols when rates of hydrolysis, dissimilar to those of simple esters, are required. Thus, an innovative use of the TML system which relied on the alkaline phosphatase-mediated hydrolysis of an aromatic phosphate derivative was employed by Ueda

* To whom correspondence should be addressed. Tel: 732-980-4924. Fax: 732-980-5911. E mail: RGREENWA@ENZON.com.

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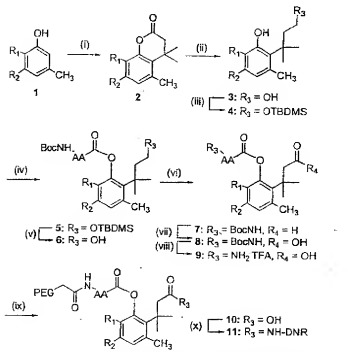
to produce a prodrug of paclitaxel (a secondary alcohol) which demonstrated comparable activity to paclitaxel in a M109 mouse model.[14] Another unique prodrug design incorporating ganciclovir (a primary alcohol) and the TML system was shown to provide 5 times greater oral bioavailability of the drug in mice.[15]

The enhanced permeation and retention (EPR) effect has been established as a useful property with which to target anticancer drugs.[16] The underlying physiological mechanism appears to be a combination of increased tumor vascular permeability with insufficient lymphatic drainage resulting in the greater accumulation of macromolecular drugs. Thus, passive accumulation of high molecular weight poly(ethylene glycol) (PEG) drug conjugates in the interstitial tissue of tumors translates into lower systemic toxicity while efficacy is enhanced, thereby increasing the therapeutic index of the drug. PEG-conjugated prodrugs (transport forms) based on 1,4- or 1,6-elimination reactions (BE system) have been demonstrated to be an effective means for delivery of the anticancer agent daunorubicin (DNR)[17] and in general provide a practical approach for solubilizing and transporting amino-containing antitumor agents as well as a variety of other drugs. In continuing our efforts to define the limits of the PEG prodrug strategy, it was apparent that the use of lactonization reactions could be incorporated into the strategy and would provide a practical alternative to elimination reactions. We have now extended and refined existing PEG technology to embrace the concept of the TML tripartate or double-prodrug system (Figure 1). To utilize the TML system for polymer-conjugated prodrugs it was necessary to first establish various methodologies which allowed the efficient synthesis of different acyl functionalities (triggers) such as esters, carbonates, and carbamates on the phenolic hydroxyl group. The acylating agents must by necessity be bifunctional and offer a site for easy PEGylation. Thus, introduction of large molecular weight PEG into the TML system as part of the specifier or trigger results in a neutral and highly water-soluble tripartate polymeric prodrug capable of passive tumor targeting. The PEG prodrug can be designed to attain predictable rates of hydrolysis by changing the nature of the trigger/linker bond, by adding steric hindrance on the aromatic ring of the linker, and by the use of different spacer groups (Figure 1). This approach offers a versatile methodology for easily altering the final design of the prodrug: it enables a "mix and match" of spacers, triggers, and linkers that can be utilized in a meaningful manner and ultimately provides optimal pharmacokinetics for delivery of different types of drugs in addition to antitumor agents.

Acylation of the primary amino group of DNR results in loss of activity of the anthracycline drug.[19] Therefore, antitumor activity can be monitored as a function of cleavage of the DNR amide prodrug conjugate in the current PEG-TML development strategy, regardless of which TML specifier cleaves.

Another drug that readily lends itself to the current prodrug strategy is the antimetabolite 1-β-D-arabinofuranosylcytosine (ara-C, cytarabine), a drug containing a weakly basic aromatic amine which is used to treat leukemia. Ara-C derivatization, as an amide, prevents rapid inactivation via cytosine deaminase conversion of

Scheme 1. Synthesis of TML Prodrugs with Ester Triggers[a]



[a] (i) Methyl 3,3-dimethylacrylate, $MeSO_3H$; (ii) LAH_4; (iii) TBDMS-Cl; (iv) EDC, DMAP, Boc-AA-OH; (v) HOAc; (vi) PCC; (vii) $NaClO_2$, H_2O_2; (viii) TFA; (ix) T-PEG, DIEA; (x) DNR·HCl, EDC, HOBT, NMM.

the N-4 amino to N-4 hydroxy and formation of inactive ara-U.[20] This was selected as a second model for modification using amino conjugation to a TML system at the N-4 position in order to demonstrate path A chemistry (see below).

This paper illustrates the versatility of the TML system using both DNR and ara-C. Synthesized PEG–DNR derivatives were subsequently evaluated in vitro and in vivo to examine their hydrolysis and biological activity, respectively. The biological effects of PEG–ara-C prodrug derivatives in the nude mouse bearing human xenografts will be the subject of a separate report.

Chemistry

Abbreviations: A (R_1 = H, R_2 = CH_3), B (R_1 = CH_3, R_2 = H), a (AA = alanine), p (AA = proline), β (AA = β-alanine) [e.g. compound **5Aa**: R_1 = H, R_2 = CH_3, AA = alanine], DCM (dichloromethane), DIEA (diisopropylethylamine), DIPC (1,3-diisopropylcarbodiimide), DNR (daunorubicin), EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), LAH (lithium aluminum hydride), HOBT (1-hydroxybenzotriazole), NMM (*N*-methylmorpholine), TBDMS-Cl (*tert*-butyldimethylsilyl chloride), TEA (triethylamine).

Ester-Based Triggers. The first entry into a hydrolytically labile PEG-TML system was accomplished using the key intermediate **8**, generated by the convenient multistep synthesis developed by Borchardt and co-workers[11] (Scheme 1). Using **6Aa** as a starting point to build a cyclic prodrug, Borchardt accomplished the elegant synthesis of a releasable peptide. For our purposes, the alaninate ester intermediate **9Aa** worked admirably as the focal point for attachment of PEG as shown in Scheme 1. When it was desired to alter the

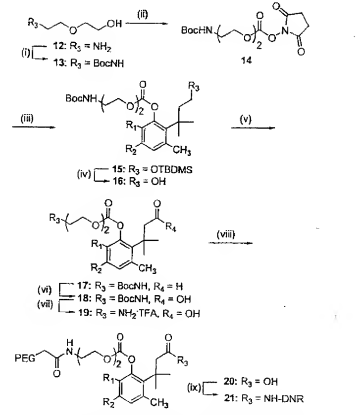
plasma half-life ($t_{1/2}$) of the PEG prodrug, the phenolic derivative **3** (Scheme 1) allowed the facile insertion of other Boc-protected amino acids such as β-alanine, or the more sterically hindered proline. In the course of this work it was found that coupling of **3** with Boc amino acids using EDC was more easily achieved than the procedure employing Boc amino acid-activated esters.[11]

Introduction of Steric Hindrance by Modification of the Ring. As described in our earlier work on drug delivery using the BE system,[17] another effective method which allowed adjustment of the rate of hydrolysis of the phenolic latentiating moiety (trigger) was to introduce additional hindering alkyl groups into the aromatic ring at the *ortho* position. This was accomplished for the TML system as depicted in Scheme 1. The known lactone derivative **2B** was synthesized starting from 2,5-dimethylphenol (**1B**) and further converted to the aldehyde **7B** in the usual manner employing pyridinium chlorochromate (PCC). Aldehyde **7B** was partially purified by simple silica gel filtration and used directly in the next step. By employing $NaClO_2$ as the oxidizing agent,[21] a high yield of pure acid **8B** was easily obtained. Additional *ortho* position modification, which substantially increased $t_{1/2}$, was utilized with different spacers and triggers (promoieties) leading to a "mix and match" situation to achieve adjustments in plasma hydrolysis. This is illustrated in Table 1 where $t_{1/2}$ and tumor efficacy of various PEG-TML prodrugs can be seen to reflect the various steric (and electronic) environments.

Carbonate-Based Triggers. Carbonate triggers for tripartate TML systems are unknown. Coupling of activated PEG (SC-PEG) directly to the phenolic OH of **4** to provide a carbonate bond was not attempted because of potential concern for degradation of the polymer during subsequent oxidation steps employing PCC. The synthesis of this type of trigger ultimately led to the use of a heterobifunctional spacer which was found to provide the necessary means to attach PEG. The design of the spacer chosen reflected an unambiguous modification since it has been previously demonstrated that anchimeric assistance to hydrolysis of esters and carbonates can occur when NH groups are placed in the δ and ϵ positions to the carbonate carbonyl.[22] The desired PEG-TML compound was obtained using the series of reactions outlined in Scheme 2. Starting with the inexpensive and readily available aminoethoxy alcohol (**12**), the activated succinimidyl carbonate **14** was prepared in 95% overall yield in two steps. Condensation of **14** with **4** gave the key TML silyl intermediate **15** which was readily converted to the amino derivative **19**. Conversion of **19** to the key PEG acid **20** was achieved using PEG thiazolidinethione (T-PEG)[23] via path B (discussed below). T-PEG is a particularly useful reagent for acylation of amines and phenols, in high yield, under mild conditions.[17] In the case of DNR, coupling to **20** was carried out with EDC in the presence of 1-hydroxybenzotriazole (HOBT) and the base *N*-methylmorpholine (NMM) to yield the desired PEG transport form (prodrug), **21**. HOBT couplings[11] were found to produce higher yields and cleaner products than other commonly used reagents such as DIPC or EDC.

Carbamate-Based Triggers. Our previous work identified a carbamate specifier (trigger) as a desirable

Scheme 2. Synthesis of TML Prodrugs with Carbonate Triggers[a]

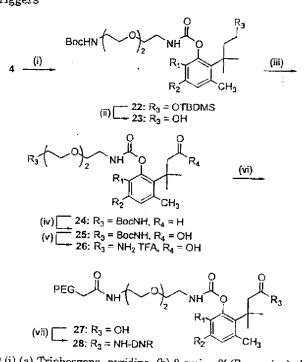


[a] (i) Boc_2O, NaOH; (ii) DSC, pyridine; (iii) **4**, DIEA; (iv) HOAc; (v) PCC; (vi) $NaClO_2$, H_2O_2; (vii) TFA; (viii) T-PEG, DIEA; (ix) DNR·HCl, EDC, HOBT, NMM.

functionality in the design of PEG–DNR prodrugs.[17] This relatively stable trigger, like the carbonate, has not yet been incorporated into TML tripartate prodrugs. We therefore devised a synthetic strategy based on the known monoprotected diamine, 2-amino-2′-(Boc-amino)-ethylene glycol diethyl ether,[24] which was condensed with the chloroformate of **4**, generated in situ using triphosgene, to give the desired carbamate **22** in 78% yield (Scheme 3). The usual sequence of reactions (**22**–**27**, Scheme 3) gave the PEG linker **27**, which was coupled to DNR mediated by EDC/HOBT/NMM. The final PEG–DNR conjugate **28** was purified by crystallization from 2-propanol.

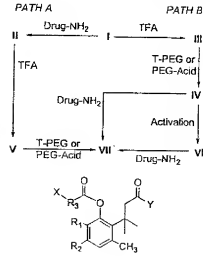
Determination of Pathway. The use of amino acid esters as bifunctional spacers enables facile attachment of the TML system to PEG. As illustrated in Scheme 4 there are two viable routes, path A and path B, that can be employed and lead to the final PEG prodrugs (**VII**). Both routes involve trifluoroacetic acid (TFA) cleavage of a Boc protecting group and generation of free amine, but path A introduces the drug first followed by PEG conjugation, while path B involves an initial attachment to PEG and subsequently bonding the drug. Path B is applicable for all candidates, but if the drug chosen to be modified can survive TFA treatment, path A is preferred because purification of the monomeric adduct prior to introduction of PEG is a more facile process and enables very high loading of parent drug in the PEG conjugates to be realized. Drug can be attached to the TML acid I by a number of methods: the simplest of these is to directly couple with a carbodiimide, usually EDC.

Scheme 3. Synthesis of TML Prodrugs with Carbamate Triggers[a]



[a] (i) (a) Triphosgene, pyridine, (b) 2-amino-2′-(Boc-amino)ethylene glycol diethyl ether, pyridine; (ii) HOAc; (iii) PCC; (iv) $NaClO_2$, H_2O_2; (v) TFA; (vi) T-PEG, DIEA; (vii) DNR-HCl, EDC, HOBT, NMM.

Scheme 4. Synthetic Routes to PEG-TML Prodrugs



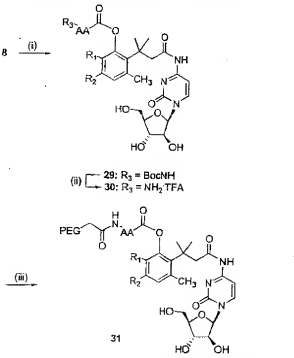
R_1 = R_2 = H or CH_3, R_3 = Spacer Moiety

I: X = BocNH, Y = OH
II: X = BocNH, Y = NH-Drug
III: X = TFA·H_2N, Y = OH
IV: X = PEG-CH_2C(=O)NH, Y = OH
V: X = TFA·H_2N, Y = NH-Drug
VI: X = PEG-CH_2C(=O)NH, Y = Z
VII: X = PEG-CH_2C(=O)NH, Y = NH-Drug

where Z = Leaving Group
(e.g. NHS, OPNP, 2-Thiazolidine thione, etc.)

Path A: Ara-C is a drug which exemplifies the facility of using this route. Usually, ara-C acylation is a low-yield reaction.[20] Condensation of TML system **8** with ara-C in the presence of EDC and pyridine gave adduct

Scheme 5. Synthesis of PEG-TML–Ara-C Prodrugs[a]



[a] (i) Ara-C, EDC, HOBT, pyridine; (ii) TFA; (iii) T-PEG, DIEA.

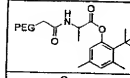
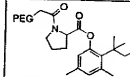
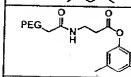
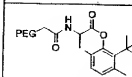
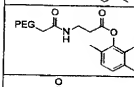
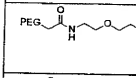
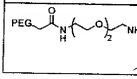
29 in the expected modest yield (60%; Scheme 5). Purification of **29** was accomplished by silica gel column chromatography followed by treatment with TFA which removed the Boc protecting group and freed the α-amino group of the alanine connector. Last, TFA salt **30** was conjugated to PEG by employing T-PEG to give the desired conjugate **31** in 84% weight yield after recrystallization from 2-propanol. On the basis of the quantitation (UV method) of ara-C in the conjugate, the conjugation yield of active drug was approximately 90%.

Path B: Treatment of DNR with TFA appears to result in cleavage of the *O*-glycosidic bond and loss of the crucial amino sugar.[25] Therefore, by necessity path B (Scheme 4) must be the synthetic route utilized in this particular case. This is illustrated in Scheme 1 where cleavage of **6** with TFA was done initially to provide **9**, followed by reaction with T-PEG to afford the key intermediate **10**. PEG acid **10** can be converted directly to the final product **11** by reaction with DNR in the presence of EDC/HOBT using DCM as the solvent. However, this procedure is not always the method of choice for other drugs.[25] Several easily prepared activated derivatives, **VI** (Scheme 4), X = *p*-nitrophenyl (PNP) and *N*-hydroxysuccinimide (NHS) esters, or the activated imide formed from the in situ generation of acid chloride followed by reaction with 2-mercaptothiazoline in the presence of a tertiary amine[23] were often utilized effectively as alternate reagents to accomplish drug conjugation when carbodiimide-mediated couplings of acids to amines were inefficient.

Results and Discussion

The synthetic methodology of the TML strategy accomplished in this work resulted in a broad, utilitarian PEG technology platform consisting of many variable combinations of specifiers and linkers for designing PEG amino prodrugs. A noteworthy feature of the TML

Table 1. In Vitro and in Vivo Results of PEG-TML Prodrugs[a]

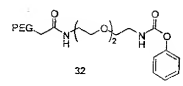
Compound	#	$t_{1/2}$ (h) Buffer pH 7.4	$t_{1/2}$ (h) Rat Plasma	$t_{1/2}$ (h) Cell Media	IC_{50} (nM) P388/O	M109 (%T/C)[b] i.p.	M109 (%T/C)[b] i.v.
Daunorubicin·HCl	-	-	-	-	3	44.8	117.0
	**11Aα**	>24	1.9	14	43	62.8	92.5
	**11Aβ**	>24	17	32	301	153.1	122.6
	**11Aβ**	>24	0.2	80	203	101.2	63.7
	**11Bα**	>24	21	36	389	153.6	72.5
	**11Bβ**	>24	8	94	411	114.8	31.6
	**21A**	>24	1.1	38	142	57.1	118.4
	**28A**	>24	>24[c]	53	203	110.5	93.8

[a] All in vitro experiments were done at 37 °C in duplicate. Standard deviation of measurements = ±10%. [b] 3 mg/kg/dose of active DNR administered to *balb/c* mice bearing subcutaneous Madison lung carcinoma on 1 & 4 (intraperitoneal) or 3 & 6 (intravenous) days after inoculation. Percent treatment over control (% T/C) median tumor volumes were compared when control groups median tumor volume reached ~2000 mm^3. [c] All the $t_{1/2}$ values were calculated from the α phase except that for **28A** which did not show a clear separation of α and β phases.

prodrugs is that they are amides and as such can be distinguished from the BE pathway which is based on carbamates. Interestingly, in the BE series a carbamate derivative of ara-C proved to be difficult to prepare while TML amide derivatives were easily obtained; thus the two methods appear to complement each other. In the present study incorporating PEG and TML linkers, a series of amino acid ester, carbonate, and carbamate triggers were prepared and integrated into a DNR–PEG-TML double prodrug. All these compounds demonstrated prolonged stability (> 24 h) in pH 7.4 phosphate buffer at 37 °C: thus, physically they have potential clinical utility as injectable agents.

Introduction or removal of substituents, either on the spacer or on the aromatic linker, resulted in significant changes of $t_{1/2}$ in rat plasma. Some interesting observations regarding plasma hydrolysis can be gleaned by examining Table 1. First, changing the alanine spacer (**11Aα**) to a proline (**11Aβ**) caused about a 10-fold decrease in the rate of hydrolysis in rat plasma, while removal of the α-substituent from spacer **11Aα** resulted in a 10-fold rate enhancement for compound **11Aβ**. Although a more rapid release rate is not desirable for severely toxic anticancer drugs, it may be useful for the rapid release of other classes of compounds. Thus the steric requirements of the spacer group are of paramount importance in the design of TML cyclization prodrugs. Interestingly, it has been reported that steric bulkiness of the acyl group (trigger) has only a very minor effect on the half-lives of esterase-mediated release of amines from model cyclization prodrugs that are coumarin based.[12c] Second, comparing **11Aα** to **11Bα** shows that introduction of an *o*-methyl substituent into the linker decreases the rate of plasma hydrolysis over 10-fold. Third, a carbonate trigger (promoiety), **21A**, hydrolyzes about 5-fold slower than an ester promoiety

Chart 1



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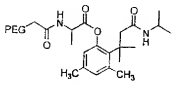

33Aa

(**11A**β). Furthermore, the hydrolysis of a carbamate trigger (**28A**) with a TML linker was unexpectedly quite slow ($t_{1/2}$ > 24 h). In the case of a similar type carbamate ester of a phenol in the BE system,[17] a much shorter $t_{1/2}$ of 4 h was found which reflected the expected mechanism of the reaction (base removal of a proton).[26] To help clarify whether the innate steric hindrance (*o-tert*-alkyl group) of the TML linker was responsible for this difference, we prepared the corresponding simple PEG linker **32** (Chart 1) based solely on phenol. Compound **32** exhibited a plasma $t_{1/2}$ = 5.2 h, thus demonstrating that *ortho* substitution may affect proton abstraction in the crowded TML system. Finally, of the seven compounds examined in Table **1**, rat plasma hydrolysis data shows only one derivative with a $t_{1/2}$ between 2 and 17 h. While further combinations of triggers and linkers should produce more intermediate $t_{1/2}$ values, it is evident that adjusting $t_{1/2}$ of TML linkers is not as facile as was the case for the BE system.

It has been our experience with PEG prodrugs that a series usually shows a direct correlation between rapid plasma $t_{1/2}$ and low IC_{50} values. For example, PEG prodrugs based on the BE system generally exhibited low IC_{50} values when their plasma $t_{1/2}$ vlaues were short indicative that hydrolysis of the promoiety also takes place in cell media.[17] However, with the exception of **11Aa**, compounds within the TML series do not appear to readily break down in cell media and subsequently had high IC_{50} values (Table 1). Fortunately, M109 results clearly established that these same compounds are indeed biologically active. This finding again demonstrates that in vitro results can be misleading in predicting in vivo outcomes. This is the first time we have observed this type of stability in cell media for a PEG prodrug series; we are currently exploring this enigma.

The safety and efficacy of the PEG conjugates within the M109 model varied according to the route of administration and the rate of in vitro dissociation (Table 1). When the compounds were dosed intraperitoneally (ip), the highest activity was observed for native DNR followed by **21A** and **11Aa**. Similar efficacy for DNR using ip dosages in this model has been reported by others.[27] However, both free DNR and **21A** were also toxic when given by this route, with DNR causing lethality in 17% of the animals while **21A** resulted in 66% lethality. In addition, **11A**β produced fatalities in 33% of the animals. Not surprisingly the three compounds that caused lethality displayed the most rapid plasma hydrolysis. However, when these compounds were administered by the more clinically relevant intravenous (iv) route, at the same dosage, they were not toxic, suggesting rapid elimination of nascent DNR. In fact, at the given dosage, none of the compounds appeared toxic when administered iv, and compounds **11A**β, **11Ba**, and **11B**β demonstrated the greatest efficacy as compared to native DNR. Interestingly, the in vitro plasma $t_{1/2}$ for the active compounds ranged from 0.2 to 24 h; thus understanding their efficacy in relation to their rates of hydrolysis is problematic and warrants an examination of their in vivo biodistribution. However, a common thread that runs through these derivatives, as well as for **11Aa** which is also quite effective, is the incorporation of an amino acid ester spacer. The production of amino acid-conjugated species has been implicated in enhanced cellular uptake of DNR.[28] Intriguingly, two recent papers have reported on the cellular uptake of amino acid ester prodrugs by a peptide transporter, although no peptide bond is present in their structure.[29,30] Further SAR relationships are currently being explored in hopes of more clearly defining the role of the spacer group in the drug delivery of antineoplastic agents.

We previously reported[17] that the type 1 carbamate derivative of DNR had an in vitro plasma $t_{1/2}$ of 4 h and produced the best solid tumor (M109 and SKOV3) results within the BE series. To compare the activities of the BE and TML compounds, the TML derivative **11Aa** appeared to approximate the BE type 1 carbamate most closely ($t_{1/2}$ of ca. 2 h) and was chosen to evaluate chemotherapeutic activity against a panel of human tumor xenografts, including SKOV3 (Table 2). SKOV3 tumors were the most sensitive to compounds in the TML series; activity was exceptionally good and quite similar to that for the BE derivative (%T/C 11 vs 5 for BE and TML, respectively). PEG conjugation did not appear to enhance the activity of DNR against tumor lines, which were insensitive to DNR (MX-1 and PC-3). Since significant antitumor activity was observed in the SKOV3 tumor line (which was susceptible to DNR treatment) we are currently planning to screen the entire TML–DNR series against an ovarian tumor panel.

To exclude the possibility that any phenolic or lactone species generated from the linker during the breakdown of the PEG–drug conjugates possessed toxic properties that might in any way affect the models, or was in part responsible for any of the tumor regression observed, we synthesized the simple isopropylamine conjugate of **10**, compound **33Aa** (Chart 1). This model compound (**33Aa**), which contained no anticancer agent, was tested in normal mice at 3 times the concentration of PEG linker used for the DNR-conjugated compound **11**. No adverse effects or toxicities were noted at this dose. Since this TML latentiated simple amine also demonstrated no in vitro cell inhibitory activity, it appears that the anticancer agent and its delivery to the tumor target are entirely responsible for any in vivo tumor growth inhibitions observed.

Conclusions

PEG conjugated to amino prodrugs that function via a TML lactonization has been demonstrated to be a

Table 2. Efficacy Comparison between DNR and PEG–DNR (11Aa) Against Subcutaneous Human Tumors[a] in Nude Mice

	DNR			PEG–DNR (11Aa)		
tumor model[b]	% tumor growth[c] (Δ from initial)	tumor inhibition[d] (%T/C)	growth delay[e] [%(T − C)/C]	% tumor growth (Δ from initial)	tumor inhibition (%T/C)	growth delay [%(T − C)/C]
MX-1 mammary carcinoma	1042	84	9	1671	124	0
PC-3 prostate adenocarcinoma	1878	89	40	2176	84	44
SKOV3 ovarian adenocarcinoma	3008+	35	16	79+*	5	>62[f]

[a] Mean baseline (initial) tumor volume was 75 mm^3. [b] 3 mg/kg/dose (DNR content) intravenously on days 1, 5, & 9. [c] % mean tumor volume change from initial based on individual tumors at weeks 5, 4, and 6 in MX-1, PC-3, and SKOV3, respectively. [d] The median tumor volumes of treatment and control groups were measured and compared when the control group's median tumor volume reached ~1000 mm^3. [e] % by which the treated median tumor volume was delayed in reaching 1000 mm^3 as compared to the control. [f] Measurements discontinued after day 60. + Significant versus control ($P < 0.05$). *Significant versus DNR ($P < 0.05$).

feasible methodology to deliver drugs—especially anticancer agents—and is comparable to the BE system[17] for introducing different trigger chemistries, spacer groups, and steric hindrance in the linker portion in order to modify rates of drug release. It offers, in some cases, the advantage of forming amides rather than carbamates. By using the double-prodrug strategy and changing promoieties (specifiers or triggers), alteration of PEG prodrug pharmacokinetics can be accomplished leading to greater drug efficacy. In a tripartate system this can be achieved not only by changing the PEG specifier or trigger but also by adding a spacer and/or introducing steric hindrance either on the spacer or on the linker, both of which will affect the rate of cleavage of the specifier.

It has been pointed out in previous work[17] that most amine drugs can be solubilized as acid salts, but their rate of renal excretion is also high. When converted to neutral small prodrug species, the ability to form salts is lost, and solubility may again become problematic. This is not so in the case of PEG–drug conjugates, where PEG confers water solubility to insoluble small organic compounds without the need for forming salts. PEG-TML prodrug methodology can be accomplished in a facile and reproducible manner, thus extending the PEG prodrug strategy for amino-containing anticancer compounds to drug release based on cyclization. These results are also anticipated to be useful for drug delivery of other difficulty soluble amine-containing pharmaceutical agents. Additional representative details will be reported soon. While we have not correlated rat plasma $t_{1/2}$ with in vivo efficacy for TML derivatives as favorably as in our previous work, the PEG-TML drug delivery approach ultimately resulted in the synthesis of a highly potent conjugate derived from DNR that appeared even more efficacious (on an equimolar basis) in an SKOV3 xenograft model than the parent drug or the BE system type 1 carbamate PEG–DNR prodrug. Thus we conclude that TML lactonization indeed offers a very practical approach for PEG modification and delivery of drugs.

Experimental Section

1. Chemistry. General. For the reasons previously described,[17] all PEGs used in this study had a molecular weight (MW) of 40 kDa. In addition, all reactions were run under an atmosphere of dry nitrogen or argon. Commercial reagents were used without further purification. DNR·HCl was obtained from Hande Tech USA, Inc. (Houston, TX) and ChemWerth (Woodbridge, CT), ara-C from Sigma Chemical Co. (Madison, WI), and PEG diol (40 kDa) from Serva (Crescent Chemical Co., NY). Boc-protected amino acids were purchased from Advanced ChemTech (Louisville, KY). All PEG compounds were dried under vacuum or by azeotropic distillation (toluene) prior to use. ^{1}H NMR spectra were obtained with a JEOL FT NMR System JNM GSX-270 instrument using deuteriochloroform as solvent unless specified. ^{13}C NMR spectra were obtained at 67.80 MHz on the JNM GSX-270. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and coupling constants (J values) are given in hertz (Hz). TLC was performed on Whatman K6F silica gel 60 Å plates. Merck silica gel 60 (230–400 mesh) was used for column chromatography. Elemental analyses were performed by Quantitative Technologies, Inc. (Whitehouse, NJ) and mass analyses (FS and HRMS) were done at Yale Cancer Center Mass Spectrometry Resource & W. M. Keck Roundation Biotechnology Resource Laboratory (New Haven, CT) and The Center for Advanced Food Technology at Rutgers, The State University of New Jersey (New Brunswick, NJ). All PEG-conjugated DNR compounds were dissolved (~15 mg/mL) in sterile saline (0.9%) for injection prior to in vivo drug treatments and were given as their DNR equivalents (absolute amount of DNR given).

HPLC Method. Analytical HPLCs were performed using a C8 reversed-phase column (Beckman, ultrasphere) under isocratic conditions with an 80:20 mixture (v/v) of methanol–water as mobile phase. Peak elutions were monitored at 254 nm using a UV detector. To detect the presence of any free PEG and also to confirm the presence of PEGylated product, an evaporative light scattering detector (ELSD), model PL-EMD 950 (Polymer Laboratories), was employed. Based on ELSD and UV analysis, all the final PEGylated products were free of native drug and were ≥95% pure by HPLC.

Analysis of DNR Content in PEG Derivatives.[31] The UV absorbance of native DNR in 86% EtOH was determined at 475 nm for six different concentrations ranging from 0.02 to 0.08 μmol/mL. From the standard plot of absorbance vs concentration, the absorption coefficient, ϵ, for DNR was calculated to be 21.6 (OD at 475 nm for 1 mg/mL with 1.0 cm light path). PEGylated DNR derivatives were dissolved in DMF at an approximate concentration of 0.015 μmol/mL (based on a MW of 40 kDa) and the UV absorbance of these compounds at 475 nm was determined. Using this value and employing the absorption coefficient, ϵ, obtained from the above, the concentration of DNR in the sample was determined. Dividing this value by the sample concentration provided the percentage of DNR in the sample.

Analysis of Ara-C Content in PEG Derivatives. For the determination of the ara-C content in PEG derivatives, N^4-acetylcytidine was used as the basis because of the absorbance change due to the acylation of ara-C. The UV absorbance of N^4-acetylcytidine in H_2O was determined at 257 nm for six different concentrations ranging from 0.01 to 0.05 μmol/mL. From the standard plot of absorbance vs concentration, the absorption coefficient, ϵ, of N^4-acetylcytidine was calculated to be 36.4 (OD at 257 nm for 1 mg/mL with 1.0 cm light path). PEGylated ara-C derivatives were dissolved in H_2O at an approximate concentration of 0.015 μmol/mL (based on a MW

of 40 kDa) and the UV absorbance of these compounds at 257 nm was determined. Using this value and employing the absorption coefficient, ϵ, obtained from the above, the concentration of ara-C in the sample was determined. Dividing this value by the sample concentration provided the percentage of ara-C in the sample.

Determination of Rates of Hydrolysis of PEG Prodrugs. The rates of hydrolysis were obtained by employing a C8 reversed-phase column (Zorbax SB-C8) using a gradient mobile phase consisting of (a) 0.1 M triethylammonium acetate buffer and (b) acetonitrile. A flow rate of 1 mL/min was used, and chromatograms were monitored using a UV detector at 254 nm for DNR and 280 nm for ara-C. For hydrolysis in buffer, PEG derivatives were dissolved in 0.1 M pH 7.4 PBS at a concentration of 5 mg/mL, while for hydrolysis in plasma, the derivatives were dissolved in distilled water at a concentration of 20 mg/100 μL and 900 μL of rat plasma was added to this solution. The mixture was vortexed for 2 min and divided into 2-mL glass vials with 100 μL of aliquot/vial. The solutions were incubated at 37 °C for various periods of time. A mixture of methanol−acetonitrile (1:1, v/v, 400 μL) was added to a vial at the proper interval and the mixture was vortexed for 1 min, followed by filtration through 0.45-mm filter membrane (optionally followed by a second filtration through 0.2-mm filter membrane). An aliquot of 20 μL of the filtrate was injected into the HPLC. On the basis of the peak area, the amounts of native compound and PEG derivative were estimated, and the half-life of each compound in different media was calculated using linear regression analysis from the disappearance of PEG derivative.

4,4,5,8-Tetramethyl-3,4-dihydrocoumarin (2B). This compound was prepared by a modification of the existing procedure:[18] 2,5-Dimethylphenol (39.4 g, 0.33 mol) was added to methanesulfonic acid (47 mL) followed by the addition of methyl 3,3-dimethylacrylate (41 g, 0.36 mol). The mixture was heated to 70 °C for 24 h. After cooling to room temperature, the solution was poured into 1 L of water. The mixture was extracted with ethyl acetate (1.5 L). The organic layer was washed with 5% $NaHCO_3$ and brine (1 L) and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo, and hexane added to the residue to precipitate a pale white solid which was collected by filtration and washed with hexane to give 32 g (48%) of product: ^{1}H NMR δ 1.38 (s, 3H, C(CH_3)$_2$), 1.44 (s, 3H, C(CH_3)$_2$), 2.26 (s, 3H, ArCH_3), 2.46 (s, 3H, ArCH_3), 2.58 (s, 2H, CH_2), 6.82 (d, J = 6.75, 1H, ArH), 6.97 (d, J = 6.75, 1H, ArH'); ^{13}C NMR δ 16.09, 22.91, 27.47, 35.19, 45.46, 124.34, 127.86, 128.98, 129.35, 133.40, 137.62, 168.18.

3-(2′-Hydroxy-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (3B). A solution of **2B** (30 g, 0.15 mol) in anhydrous THF (300 mL) was cooled in an ice bath and slowly added to a suspension of LAH (10.0 g, 0.285 mol) in THF (150 mL) under nitrogen. The mixture was stirred overnight 0 °C and then allowed to warm to room temperature. The reaction was monitored by TLC. 300 mL of HPLC grade THF was added slowly with condenser attached, followed by 40 mL of saturated aqueous ammonium chloride solution to quench excess LAH, and the mixture was filtered. The solid was washed with THF (1.5 L) and the solvent was removed in vacuo from the filtrate. The residue was dissolved in DCM (700 mL) and washed with water (2 × 150 mL). The organic layer was dried over anhydrous $MgSO_4$ and the soivent removed in vacuo. The residue was purified by column chromatography (10−20% EtOAc in toluene) to give 23 g (75%) of product: ^{1}H NMR δ 1.55 (s, 6H, 2 × C(CH_3)$_2$), 2.14 (s, 3H, ArCH_3), 2.20 (t, J = 6.75, 2H, CH_2CH_2OH), 2.46 (s, 3H, ArCH_3), 3.52 (t, J = 6.75, 2H, CH_2CH_2OH), 5.70 (s, 1H, OH), 6.56 (d, J = 6.75, 1H, ArH), 6.83 (d, J = 6.75, 1H, ArH'); ^{13}C NMR δ 16.22, 22.02, 31.95, 39.78, 44.81, 61.06, 122.23, 125.46, 127.86, 131.82, 135.63, 153.60; EI MS m/z 208 (M^+, 50); HRMS calcd for $C_{13}H_{20}O_2$ (M^+) 208.1462, found 208.1463.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-hydroxy-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (4B). Compound **3B** (23 g, 0.11 mol) and TBDMS-Cl (18.6 g, 0.12 mol) were dissolved in DCM (140 mL) at 0 °C and a solution of TEA (62 mL) in DCM (60 mL) was added dropwise over 1 h. The mixture was warmed to room temperature and stirred overnight. The solvent was removed at 40 °C in vacuo and the residue dissolved in DCM (500 mL). The solution was washed with water (4 × 50 mL) and dried over anhydrous $MgSO_4$. The solvent was removed to give 32 g (91%) of product: ^{1}H NMR δ 0.08 (s, 6H, Si(CH_3)$_2$), 0.86 (s, 9H, SiC(CH_3)$_3$), 1.56 (s, 6H, C(CH_3)$_2$), 2.14 (t, J = 6.75, 2H, CH_2CH_2OSi), 2.15 (s, 3H, ArCH_3), 2.45 (s, 3H, ArCH_3), 3.59 (t, J = 6.75, 2H, CH_2 CH_2-OSi), 5.80 (s, 1H, OH), 6.55 (d, J = 6.75, 1H, ArH), 6.82 (d, J = 6.75, 1H, ArH'); ^{13}C NMR δ 16.30, 18.19, 25.51, 25.62, 25.87, 25.87, 21.21, 39.67, 44.94, 61.66, 122.72, 125.18, 127.71, 132.04, 135.38, 153.79.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-Boc-prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (5Ap). DIPC (392 mg, 3.11 mmol) was added to a mixture of **4A**[19] (0.5 g, 1.55 mmol), DMAP (568 mg, 4.66 mmol), and Boc-Pro OH (668 mg, 3.11 mmol) in anhydrous DCM (15 mL) at 0 °C. The mixture was stirred at room temperature overnight, then filtered, and concentrated. The residue was purified using silica gel column chromatography (ethyl acetate−hexane = 3:7, v/v) to give 700 mg (87%) of **5Ap**: ^{1}H NMR δ 0.04 (s, 3H, CH_3Si), 0.14 (s, 3H, CH_3Si), 0.88 (s, 3H, CH_3C), 0.92 (s, 3H, CH_3C), 1.51 (s, 9H, Si(CH_3)$_3$), 1.55 (s, 9H, C(CH_3)$_2$), 2.00 (m, 2H, CH_2O), 2.07 (t, 2H, J = 8.1, $CH_2CH_2CH_2$), 2.25 (s, 3H, PhCH_3), 2.30 (m, 2H, $CHCH_2$) 2.55 (s, 3H, PhCH_3), 3.53 (t, 2H, J = 8.1, CH_2N), 4.57 (br s, 1H, COCHN), 6.65 (bs, 1H, ArH), 6.82 (s, 1H, ArH'); ^{13}C NMR δ −4.37, 18.23, 20.06, 25.14, 25.70, 25.98, 28.49, 32.00, 32.06, 39.51, 46.24, 46.62, 59.74, 60.93, 80.08, 122.73, 132.14, 134.56, 135.87, 138.30, 159.68, 171.84; EI MS m/z 542.37 (M^+ + Na, 70), 558.34 (M^+ + K, 35); HRMS calcd for $C_{29}H_{49}NO_5$-SiNa (M^+ + Na), 542.3278, found 542.3333.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-Boc-β-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (5Aβ). EDC·HCl (35 g, 0.182 mol) was added to the mixture of **4A** (20 g, 0.062 mol), Boc-β-Ala-OH (23.5 g, 0.124 mol), and DMAP (53 g, 0.434 mol) in anhydrous DCM (150 mL) at 0 °C. The mixture was stirred overnight and 600 mL of DCM was added. The solution was washed with 1% $NaHCO_3$ (3 × 150 mL) and 1 N HCl (3 × 150 mL) and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (10% EtOAc in hexane) to give 26 g (85%) of product: ^{1}H NMR δ 0.00 (s, 6H, 2 × CH_3Si), 0.87 (s, 9H, 2 × CH_3C), 1.48 (s, 18H, C(CH_3)$_3$ & Si(CH_3)$_2$), 2.05 (t, 2H, J = 5.4, CH_2C), 2.26 (s, 3H, ArCH_3), 2.54 (s, 3H, ArCH_3), 2.79 (t, 2H, J = 5.4, CH_2C(=O)), 3.50 (t, 4H, J = 8.1, NHCH_2 & CH_2O,), 5.01 (br s, 1H, NH), 6.58 (s, 1H, ArH), 6.84 (s, 1H, ArH'); ^{13}C NMR δ −4.42, 18.18, 20.11, 25.20, 25.88, 28.36, 31.78, 35.40, 36.00, 38.99, 45.94, 60.69, 79.35, 122.91, 132.38, 133.99, 135.92, 138.40, 149.46, 155.79, 171.73. Anal. ($C_{32}H_{57}$-NO_5Si) C, H, N.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-Boc-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (5Aa). Prepared by reaction of **4B** and Boc-Ala-OH in 98% yield as described for **5Aβ**: ^{1}H NMR δ 0.03 (s, 6H, 2 × Si(CH_3)$_2$), 0.91 (s, 9H, SiC(CH_3)$_3$), 1.49 (s, 9H, OC(CH_3)$_3$), 1.54 (s, 6H, 2 × C(CH_3)$_2$), 1.61 (t, 2H, J = 6.75, CH_2CH_2OSi), 2.14 (m, 4H, CH(CH_3)$_3$ & ArCH_3), 2.45 (s, 3H, ArCH_3), 3.50 (t, 2H, J = 6.75, CH_2CH_2-OSi), 4.64 (m, 1H, CH(CH$_3$)$_3$), 5.26 (m, 1H, NH), 6.95 (m, 2H, 2 × ArH); ^{13}C NMR δ −5.38, 17.25, 18.16, 18.68, 25.22, 25.88, 28.28, 31.53, 31.88, 39.52, 45.85, 46.10, 49.54, 49.93, 60.70, 79.79, 128.51, 128.88, 131.35, 136.49, 137.52, 148.36, 154.97, 171.70.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-Boc-β-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (5Aβ). Prepared by reaction of **4B** and Boc-β-Ala-OH in 90% yield as described for **5Aβ**: ^{1}H NMR δ −0.05 (s, 6H, Si(CH_3)$_2$), 0.82 (s, 9H, SiC-(CH_3)$_3$), 1.42 (s, 9H, OC(CH_3)$_3$), 1.46 (s, 6H, 2 × C(CH_3)$_2$), 2.00 (s, 3H, ArCH_3), 2.05 (m, 2H, CH_2CH_2OSi), 2.50 (s, 3H, ArCH_3), 2.78 (m, 2H, NHCH_2), 3.50 (m, 4H, CH_2CH_2OSi & CH_2C(=O)O), 5.13 (br s, 1H, NH), 6.90 (dd, 2H, J = 13.36 & 7.76, 2 × ArH); ^{13}C NMR δ −5.45, 17.11, 18.13, 25.14, 25.84, 28.30, 31.15, 31.82, 34.81, 35.91, 39.35, 46.00, 60.62, 79.22, 128.35,

135.87, 131.15, 136.21, 137.23, 148.52, 155.72, 171.06. Anal. ($C_{27}H_{47}NO_5Si$) C, H, N.

3-(2′-Boc-prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (6Ap). A mixture of **5Ap** (2.82 g, 5.43 mmol), THF (10 mL), H_2O (10 mL), and HOAc (30 mL) was stirred at room temperature for 1 h. The solvent was removed to give the product as a colorless oil (2.2 g, 100%). The product was used without further purification: ^1H NMR δ 1.38, 1.40, 1.42, 1.93, 1.97, 2.01, 2.13, 2.38, 2.43, 3.47, 4.48, 6.50, 6.70; ^{13}C NMR δ 19.95, 20.29, 23.19, 25.06, 25.20, 25.59, 28.44, 31.78, 32.04, 32.27, 33.21, 39.33, 42.96, 46.01, 46.53, 46.62, 59.62, 60.22, 64.95, 66.59, 80.44, 103.87, 122.59, 132.04, 132.27, 134.23, 135.79, 135.89, 138.14, 150.37, 150.55, 154.52, 171.86, 175.10.

3-(2′-Boc-β-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (6Aβ). Prepared from **5Aβ** in 95% yield as described for **6Ap**: ^1H NMR δ 1.45 (s, 9H, *t*-Bu), 1.48 (s, 6H, 2 × $C(CH_3)$), 2.04 (t, 2H, J = 7.25, CH_2CH_2OH), 2.23 (s, 3H, $ArCH_3$), 2.53 (s, 3H, $ArCH_3$), 2.78 (t, 2H, J = 5.94, CH_2CH_2-NH), 3.51 (m, 4H, CH_2OH & CH_2CH_2NH), 5.13 (bs, 1H, N*H*), 6.55 (d, 1H, J = 1.97, Ar*H*), 6.83 (d, 1H, J = 1.97, Ar*H*).

3-(2′-Boc-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (6Ba). Prepared from **5Ba** in 90% yield as described for **6Ap**: ^{13}C NMR δ 17.77, 17.92, 25.48, 28.27, 31.82, 32.00, 32.08, 32.26, 39.59, 45.67, 49.54, 50.08, 60.10, 80.39, 128.54, 128.70, 128.95, 131.43, 136.21, 137.41,137.85, 148.59, 155.04, 155.56, 171.61.

3-(2′-Boc-β-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanol (6Bβ). Prepared from **5Bβ** in 90% yield as described for **6Ap**: ^{13}C NMR δ 17.07, 25.20, 25.57, 28.27, 31.99, 34.84, 35.87, 39.37, 45.68, 53.36, 60.13, 79.35, 128.48, 129.01, 131.26, 136.30, 137.08, 148.49, 155.75, 171.52.

3-(2′-Boc-prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanal (7Ap). A solution of **6Ap** (2.5 g, 6.2 mmol) in anhydrous DCM (125 mL) was slowly added to a suspension of pyridinium chlorochromate (2.88 g, 13.4 mmol) in anhydrous DCM (125 mL) at room temperature and allowed to stir overnight at room temperature. The reaction mixture was concentrated and the residue dissolved in 30 mL of DCM followed by filtration through a short (~2 in.) silica gel pad. The silica gel was flushed several times with ethyl ether. The filtrate was concentrated in vacuo to give the product as a viscous oil (2.3 g, 88%). The aldehyde was used in the next step without further purification.

3-(2′-Boc-β-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanal (7Aβ). Prepared from **6Aβ** in 90% yield as described for **7Ap**: ^1H NMR δ 1.45 (s, 9H, *t*-Bu), 1.55 (s, 6H, 2 × $C(CH_3)$), 2.24 (s, 3H, $ArCH_3$), 2.54 (s, 3H, $ArCH_3$), 2.78 (d, 2H, J = 5.94, CH_2CH_2NH), 2.81 (d, 2H, J = 2.30, CH_2CHO), 3.50 (d, 2H, J = 5.94, CH_2CH_2NH), 5.09 (br s, 1H, N*H*), 6.61 (s, 1H, Ar*H*), 6.86 (d, 1H, J = 1.97, Ar*H*), 9.53 (t, 1H, J = 2.62, C(=O)*H*).

3-(2′-Boc-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanal (7Ba). Prepared from **6Ba** in 67% yield as described for **7Ap**: ^1H NMR δ 1.45 (s, 9H, *t*-Bu), 1.61 (m, 9H, 2 × CH_3 & $CHCH_3$), 2.04 (s, 3H, $ArCH_3$), 2.54 (s, 3H, $ArCH_3$), 2.88 (ABq, J = 101.27 & 16.20, 2H, CH_2CHO), 4.59 (m, 1H, $CHCH_3$), 5.13 (m, 1H, N*H*), 6.97 (q, J = 5.4, 2H, 2 × Ar*H*), 9.53 (s, 1H, C(=O)*H*); ^{13}C NMR δ 17.21, 18.26, 25.36, 28.22, 31.40, 31.87, 38.48, 49.56, 49.98, 56.52, 56.78, 80.02, 129.19, 131.72, 135.89, 136.37, 148.02, 155.10, 171.86, 202.72.

3-(2′-Boc-β-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropanal (7Bβ). Prepared from **6Bβ** in 99% yield as described for **7Ap**: ^{13}C NMR δ 15.07, 16.97, 285.13, 25.49, 28.18, 31.45, 34.72, 35.78, 38.24, 56.51, 65.61, 79.23, 129.01, 129.24, 131.45, 135.57, 135.79, 148.06, 155.60, 170.86, 202.40.

3-(2′-Boc-prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (8Ap). A solution of 80% sodium chlorite (4 g, 0.032 mol) in water (37 mL) was slowly added to a solution of **7Ap** (5.45 g, 0.013 mol) and sodium dihydrogen phosphate, NaH_2PO_4 (0.98 g, 0.008 mol), in CH_3CN (27 mL) and water (11 mL) that had been cooled to 0 °C in an ice–salt water bath. The mixture was stirred for 1 h at 0 °C and then allowed to reach room temperature. Sodium sulfite (1.73 g, 0.013 mol) was added to the reaction to decompose HOCl and H_2O_2. The pH was adjusted to 2.0 with 1 N HCl, followed by extraction with ethyl acetate (500 mL). The organic layer was washed with brine (2 × 200 mL) and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo and the residue was purified with silica gel column chromatography (5% MeOH in DCM) to give the product **8Ap** (4.5 g, 83%): ^1H NMR δ 1.41, 1.46, 1.50, 1.52, 1.61, 1.97, 2.21, 2.30, 2.33, 2.54, 2.85, 3.56, 4.56, 6.57, 6.79; ^{13}C NMR δ 20.09, 24.13, 25.17, 28.14, 28.56, 29.87, 31.40, 31.57, 39.12, 46.79, 47.40, 59.76, 80.68, 122.56, 132.43, 134.04, 136.27, 138.29, 150.21, 171.87.

3-(2′-Boc-β-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (8Aβ). Prepared from **7Aβ** in 78% yield as described for **8Ap**: ^1H NMR δ 1.44 (s, 9H, *t*-Bu), 1.57 (s, 6H, 2 × $C(CH_3)$), 2.23 (s, 3H, $ArCH_3$), 2.54 (s, 3H, $ArCH_3$), 2.76 (d, 2H, J = 5.94, CH_2CH_2NH), 2.81 (s, 2H, CH_2COOH), 3.48 (d, 2H, J = 5.94, CH_2CH_2NH), 5.14 (br s, 1H, N*H*), 6.58 (s, 1H, Ar*H*), 6.82 (d, 1H, J = 1.97, Ar*H*); ^{13}C NMR δ 14.03, 20.07, 20.86, 25.10, 28.24, 28.52, 31.22, 35.30, 35.90, 38.50, 38.50, 47.30, 60.27, 79.80, 122.81, 132.38, 133.27, 136.05, 138.00, 149.09, 155.83, 171.07, 171.47, 176.28. Anal. ($C_{21}H_{31}NO_6$·1/2EtOAc) C, H, N.

3-(2′-Boc-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (8Ba). Prepared from **7Ba** in 95% yield as described for **8Ap**. NMR resonances showed multiplicity presumably because of the presence of two *ortho* substituents: ^1H NMR δ 1.43 (s, 9H, *t*-Bu), 1.59 (m, 9H, 2 × CH_3 & $CHCH_3$), 2.01 (s, 3H, $ArCH_3$), 2.53 (s, 3H, $ArCH_3$), 2.84 (ABq, J = 101.27 & 16.20, 2H, CH_2COOH), 4.59 (m, 1H, $CHCH_3$), 5.20 (m, 1H, N*H*), 6.92 (q, J = 5.4, 2H, 2 × Ar*H*); ^{13}C NMR δ 17.21, 18.14, 25.28, 28.27, 28.78, 31.05, 31.56, 39.06, 47.16, 47.74, 50.00, 80.02, 128.84, 131.53, 136.20, 137.17, 148.16, 155.44, 171.79, 175.81; EI MS *m/z* 416.23 (M^+ + Na, 100), 432.21 (M^+ + K, 85); HRMS calcd for $C_{21}H_{31}NO_6Na$ (M^+ + Na) 416.2049, found 416.2047.

3-(2′-Boc-β-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (8Bβ). Prepared from **7Bβ** in 78% yield as described for **8Ap**: ^{13}C NMR δ 16.95, 24.97, 28.47, 31.13, 34.66, 35.78, 38.77, 47.39, 79.23, 128.54, 128.81, 131.17, 135.76, 136.71, 148.13, 155.80, 170.95, 176.41; EI MS *m/z* 416.23 (M^+ + Na, 95), 432.19 (M^+ + K, 90); HRMS calcd for $C_{21}H_{31}NO_6$-Na (M^+ + Na) 416.2049, found 416.2046.

3-(2′-Alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9Aa). Trifluoroacetic acid (10 mL) was added to a solution of **8Aa**[11] (1.0 g, 2.54 mmol) in DCM (10 mL) and the mixture was stirred at room temperature for 2 h. Solvent was removed completely in vacuo followed by addition of ethyl ether to precipitate the product (as the TFA salt, 0.6873 g, 67%): ^1H NMR δ 1.41 (3H, s, $C(CH_3)_2$), 1.42 (3H, s, $C(CH_3)_2$), 1.54 (3H, d, J = 8.1, $CHCH_3$), 2.21 (3H, s, $ArCH_3$), 2.45 (3H, s, $ArCH_3$), 2.65 (2H, s, CH_2COOH), 4.36 (1H, d, J = 8.1, $CHCH_3$), 6.57 (1H, s, Ar*H*), 6.81 (1H, s, Ar*H*); ^{13}C NMR δ 15.27, 19.57, 24.66, 30.92, 38.28, 47.32, 48.45, 122.23, 132.30, 133.97, 135.61, 138.05, 149.10, 169.50, 172.50.

3-(2′-Prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9Ap). Prepared from **8Ap** in 77% yield as described for **9Aa**: ^1H NMR δ 1.53, 1.55, 2.03, 2.19, 2.48, 2.59, 2.76, 3.32, 4.61, 6.54, 6.85, 9.80; ^{13}C NMR δ 13.86, 23.73, 24.86, 28.27, 31.70, 31.92, 38.99, 46.43, 47.76, 60.33, 122.25, 133.08, 133.74, 136.65, 138.89, 149.30, 168.35.

3-(2′-β-Alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9Aβ). Prepared from **8Aβ** in 69% yield as described for **9Aa**: ^1H NMR ($CDCl_3$ + CD_3OD) δ 1.55 (s, 6H, 2 × $C(CH_3)$), 2.21 (s, 3H, $ArCH_3$), 2.53 (s, 3H, $ArCH_3$), 2.78 (s, 2H, CH_2CH_2NH), 2.85 (s, 2H, CH_2COOH), 3.12 (br s, 2H, CH_2CH_2NH), 6.55 (s, 1H, Ar*H*), 6.87 (s, 1H, Ar*H*), 10.19 (s, COO*H*); ^{13}C NMR ($CDCl_3$ + CD_3OD) δ 19.35, 24.44, 30.93, 31.61, 34.88, 38.21, 47.17, 122.26, 132.17, 133.20, 135.83, 137.99, 148.55, 169.91, 174.15.

3-(2′-Alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9Ba). Prepared from **8Ba** in 50% yield as described for **9Aa**: ^1H NMR (DMSO-d_6) δ 1.41 & 1.43 (s, 3H, CH_3), 1.56 (s, 3H, CH_3), 1.65 & 1.67 (d, J = 6.59, 3H, $CHCH_3$), 1.99 (s, 3H, $ArCH_3$), 2.51 (s, 3H, $ArCH_3$), 2.70 (m, 2H, CH_2COOH), 4.52 (m, 1H, $CHCH_3$), 6.98 (m, 2H, 2 × Ar*H*), 9.65 (br s, 2H, NH_2);

^{13}C NMR (DMSO-d_6) δ 15.05, 16.76, 24.57, 30.90, 38.70, 48.03, 48.58, 114.93, 119.35, 128.17, 128.44, 131.31, 135.94, 137.44, 147.31, 157.98, 158.43, 168.64, 172.37.

3-(2′-β-Alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9Bβ). Prepared from **8Bβ** in 65% yield as described for **9Aa**: ^{13}C NMR δ 14.81, 16.59, 24.81, 30.83, 31.83, 35.39, 39.23, 47.07, 65.91, 128.67, 129.12, 131.52, 136.60, 136.76, 147.94, 170.74, 175.81.

3-(2′-PEG-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (10Aa). Diisopropylethylamine (36.6 μL, 0.20 mmol) was added to a solution of **7**-PEG[23] (1 g, 0.025 mmol) and **9Aa** (29 mg, 0.099 mmol) in anhydrous DCM (15 mL) and the mixture was stirred at room temperature overnight. The solvent was removed in vacuo and the residue recrystallized from 2-propanol to give 0.8 g (80%) of product: ^{13}C NMR δ 16.90, 19.48, 24.45, 30.81, 38.34, 46.53, 47.87, 68.52–72.82 (PEG), 76.19, 77.83, 122.00, 131.77, 133.76, 135.40, 137.51, 149.46, 169.28, 170.88, 171.55.

3-(2′-PEG-prolinyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (10Ap). Prepared from **9Ap** in 85% yield as described for **10Aa**: ^{13}C NMR δ 19.61, 24.39, 27.34, 30.72, 38.11, 45.86, 59.13, 68.40–71.45 (PEG), 122.18, 131.78, 133.40, 135.04, 137.18, 149.36, 168.65, 170.67, 171.18.

3-(2′-PEG-β-alaninyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (10Aβ). Prepared from **9Aβ** in 90% yield as described for **10Aa**: ^{13}C NMR δ 19.44, 24.47, 30.70, 33.56, 34.13, 37.90, 46.62, 69.77–70.25 (PEG), 122.17, 131.47, 133.06, 134.92, 137.25, 148.54, 169.29, 170.08, 171.76.

3-(2′-PEG-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (10Ba). Prepared from **9Ba** in 87% yield as described for **10Aa**: ^{13}C NMR δ 16.25, 16.75, 24.27, 30.12, 30.51, 37.95, 46.84, 47.98, 69.51–70.30 (PEG), 127.61, 130.33, 134.94, 136.54, 147.22, 168.78, 169.64, 171.23.

3-(2′-PEG-β-alaninyl-3′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (10Bβ). Prepared from **9Bβ** in 96% yield as described for **10Aa**: ^{13}C NMR δ 16.42, 24.48, 30.70, 33.47, 38.25, 46.65, 68.17–71.90 (PEG), 127.80, 128.15, 130.49, 135.17, 136.51, 147.61, 169.29, 169.68, 171.64.

PEG-TML–DNR. Compound 11Aa. EDC·HCl (94.5 mg, 0.492 mmol) was added to a mixture of **10Aa** (2.5 g, 0.0615 mmol), DNR·HCl (208 mg, 0.3688 mmol), NMM (99.4 mg, 0.984 mmol), and HOBT hydrate (49.8 mg, 0.369 mmol) in 50 mL of anhydrous methylene chloride at 0 °C. The reaction mixture was stirred at room temperature overnight and filtered, and the filtrate was concentrated in vacuo. The residue was recrystallized from 2-propanol (200 mL) to give 2.3 g (92%) of product. The amount of DNR present in this compound as measured by UV assay was 2.4 wt %: ^{13}C NMR δ 16.14, 19.31, 23.55, 24.63, 28.80, 31.15, 32.78, 34.77, 39.23, 44.97, 48.12, 56.18, 66.80–75.95 (PEG), 100.14, 110.93, 118.68, 119.13, 121.81, 131.88, 133.86, 134.02, 134.96, 135.03, 137.96, 149.56, 155.20, 160.73, 169.73, 170.22, 171.34, 186.05, 210.41.

Compound 11Ap. Prepared from **10Ap** in 84% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.5 wt %: ^{13}C NMR δ 16.03, 19.33, 20.65, 21.57, 23.76, 24.41, 24.86, 26.96, 26.94, 27.83, 28.71, 29.89, 31.04, 31.30, 32.60, 34.38, 39.02, 44.16, 44.63, 45.85, 46.82, 55.73, 58.91, 58.97, 59.72, 66.77, 67.76–71.43 (PEG), 75.59, 77.90, 99.74, 110.11, 110.28, 117.92, 118.64, 119.46, 119.69, 120.84, 131.25, 133.46, 134.30, 134.93, 137.46, 148.39, 154.72, 155.42, 160.09, 167.99, 169.38, 170.55, 171.13, 185.53, 185.73, 205.25, 210.54.

Compound 11Aβ. Prepared from **10Aβ** in 90% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.5 wt %: ^{13}C NMR δ 16.10, 19.38, 23.92, 24.73, 28.46, 31.09, 32.46, 33.65, 34.32, 38.97, 44.36, 48.53, 55.90, 66.70–69.77 (PEG), 75.76, 100.15, 110.40, 117.98, 118.87, 122.26, 131.74, 133.62, 134.57, 135.05, 135.60, 137.62, 148.92, 154.94, 155.60, 160.26, 169.39,169.76, 170.72, 185.76, 210.97.

Compound 11Ba. Prepared from **10Ba** in 90% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.5 wt %: ^{13}C NMR δ 15.79, 15.90, 16.13, 23.57, 24.41, 24.79, 28.18, 30.99, 31.21, 32.09, 32.11, 34.13, 38.74, 44.16, 44.39, 46.82, 47.71, 47.97, 55.62, 66.06–69.50 (PEG), 75.44, 99.90, 110.12, 110.17, 117.92, 118.53, 119.63, 127.30, 127.59, 130.48, 133.40, 134.21, 134.82, 135.52, 135.93, 136.86, 147.35, 154.59, 155.11, 155.31, 155.62, 160.03, 169.03, 169.18, 170.30, 170.33, 170.94, 185.42, 185.59, 210.77.

Compound 11Bβ. Prepared from **10Bβ** in 90% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.5 wt %: ^{13}C NMR δ 16.13, 16.31, 23.97, 34.82, 28.35, 30.95, 31.61, 32.40, 32.50, 33.79, 34.28, 39.40, 44.35, 48.49, 55.88, 66.34, 67.86–75.73 (PEG), 78.28, 100.12, 110.28, 110.42, 117.93, 118.85, 119.89, 128.23, 128.43, 130.75, 133.50, 133.66, 134.53, 135.05, 135.58, 147.97, 154.92, 156.60, 160.21, 169.32, 169.64, 185.68, 186.00, 211.03.

2-(2-Boc-aminoethoxy)ethanol (13). This compound was prepared by a modification of the existing procedure.[32] A solution of di-*tert*-butyl carbonate (10.27 g, 47.2 mmol) in chloroform (40 mL) was added to a solution of 2-(2-aminoethoxy)ethanol (**12**; 5.0 g, 47.62 mmol) in chloroform (40 mL) and the mixture was stirred at room temperature for 1.5 h. The solution was washed with water (30 mL) and the organic layer dried over anhydrous $MgSO_4$ and concentrated in vacuo to give the product (9.6 g, 99%): ^{1}H NMR δ 1.45 (s, 9H, *t*-Bu), 3.32 (m, 2H, $NHCH_2$), 3.43 (bs, 1H, OH), 3.56 (m, 4H, CH_2-OCH_2), 3.73 (t, 2H, J = 5.4, CH_2OH), 4.43 (bs, 1H, NH); ^{13}C NMR δ 28.21, 40.21, 61.31, 70.08, 72.14, 77.18, 79.06, 156.06.

2-(2-Boc-aminoethoxy)ethanol NHS Carbonate (14). A mixture of **13** (1.0 g, 4.88 mmol), *N,N*-disuccinimidyl carbonate (1.5 g, 5.86 mmol), and anhydrous pyridine (474 mg, 6.0 mmol) in anhydrous chloroform (25 mL) was stirred at 25–30 °C overnight. The reaction mixture was washed with 0.5 N HCl then dried over anhydrous $MgSO_4$. Evaporation of the solvent gave the product (1.5 g, 96%): ^{1}H NMR δ 1.45 (s, 9H, *t*-Bu), 2.84 (bs, 4H, NHS), 3.32 (m, 2H, $NHCH_2$), 3.51 (t, 2H, J = 4.9, CH_2O), 3.74 (t, 2H, J = 4.3, OCH_2), 4.47 (t, 2H, J = 4.3, $CH_2OC(=O)ONHS$), 5.01 (br s, 1H, NH); ^{13}C NMR δ 25.00, 27.92, 39.81, 67.57, 69.59, 69.89, 77.20, 78.56, 151.18, 155.58, 168.60.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-2″-(2‴-Boc-aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (15A). A mixture of **14** (1.0 g, 3.13 mmol), **4A** (503 mg, 1.56 mmol), and DIEA (600 μL, 3.51 mmol) in chloroform (20 mL) was refluxed overnight. The reaction mixture was washed with 0.5 N HCl (2 × 20 mL) and dried over anhydrous $MgSO_4$ and the solvent was removed in vacuo. The residue was purified by column chromatography (10–30% ethyl acetate in hexane) to give the product (470 mg, 28%): ^{1}H NMR δ −0.07 (s, 6H, $Si(CH_3)_2$), 0.81 (s, 9H, $SiC(CH_3)_3$), 1.42 (s, 9H, *t*-Bu), 1.46 (s, 6H, $C(CH_3)_2$), 2.01 (t, 2H, J = 7.60, CH_2OSi), 2.21 (s, 3H, $ArCH_3$), 2.49 (s, 3H, $ArCH_3$), 3.30 (q, 2H, J = 5.12, $NHCH_2$), 3.46 (t, 2H, J = 7.43, CH_2CH_2OSi), 3.52 (t, 2H, J = 5.28, CH_2CH_2O), 3.70 (t, 2H, J = 4.62, OCH_2), 4.35 (t, 2H, J = 4.78, $CH_2OC(=O)O$), 4.93 (br s, 1H, NH), 6.63 (s, 1H, ArH), 6.79 (s, 1H, ArH); ^{13}C NMR δ −5.43, 18.10, 20.05, 25.03, 25.83, 28.32, 31.64, 39.11, 40.30, 45.83, 60.65, 67.16, 68.57, 70.25, 79.16, 122.44, 132.43, 134.05, 135.98, 138.27, 150.08, 154.05, 155.84. Anal. ($C_{38}H_{70}N_2O_9Si$) C, H, N.

3-(2′-2″-(2‴-Boc-aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (16A). Prepared from **15A** in 82% yield as described for **6Ap**: ^{1}H NMR δ 1.42 (s, 9H, *t*-Bu), 1.49 (s, 6H, $C(CH_3)_2$), 2.05 (t, 2H, J = 7.43, CH_2-OH), 2.22 (s, 3H, $ArCH_3$), 2.50 (s, 3H, $ArCH_3$), 3.31 (q, 2H, J = 5.28, $NHCH_2$), 3.52 (m, 4H, CH_2CH_2OH & CH_2O), 3.72 (m, 2H, OCH_2), 4.39 (m, 2H, $CH_2OC(=O)O$), 5.12 (br s, 1H, NH), 6.67 (s, 1H, ArH), 6.82 (d, 1H, J = 1.32, ArH); ^{13}C NMR δ 20.18, 25.26, 28.43, 31.87, 39.31, 40.44, 46.11, 60.48, 67.28, 68.73, 70.41, 79.23, 122.65, 132.75, 133.90, 136.38, 138.42, 150.11, 154.34, 155.99.

3-(2′-2″-(2‴-Boc-aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanal (17A). Prepared from **16A** as described for **7Ap** in 97% yield: ^{1}H NMR δ 1.40 (s, 9H, *t*-Bu), 1.54 (s, 6H, $C(CH_3)_2$), 2.21 (s, 3H, $ArCH_3$), 2.50

(s, 3H, ArCH_3), 2.82 (d, 2H, J = 2.64, CH_2CHO), 3.29 (q, 2H, J = 5.12, NHCH_2), 3.52 (t, 2H, J = 5.12, CH_2O), 3.69 (m, 2H, OCH_2), 4.36 (m, 2H, CH_2OC(=O)O), 4.96 (bs, 1H, NH), 6.69 (s, 1H, ArH), 6.82 (d, 1H, J = 1.32, ArH), 9.50 (t, 1H, J = 2.48, CH_2CHO); ^{13}C NMR δ 20.11, 25.22, 28.30, 31.48, 40.27, 56.59, 67.37, 68.49, 70.22, 79.18, 133.67, 132.56, 132.73, 136.88, 137.72, 149.68, 153.81, 155.84, 202.57.

3-(2′-2″-(2″-Boc-aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (18A). Prepared from **17A** as described for **8Ap** in 97% yield: ^{1}H NMR δ 1.42 (s, 9H, t-Bu), 1.61 (s, 6H, C(CH_3)$_2$), 2.24 (s, 3H, ArCH_3), 2.55 (s, 3H, ArCH_3), 2.86 (s, 2H, CH_2COOH), 3.31 (bs, 2H, NHCH_2), 3.56 (m, 2H, CH_2O), 3.74 (m, 2H, OCH_2), 4.40 (m, 2H, CH_2OC(=O)O), 5.06 (bs, 1H, NH), 6.72 (s, 1H, ArH), 6.83 (s, 1H, ArH); EI MS m/z 476.22 (M$^+$ + Na, 95), 492.21 (M$^+$ + K, 90); HRMS calcd for $C_{25}H_{40}N_2O_8Na$ (M$^+$ + Na) 476.2260, found 476.2278.

3-(2′-2″-(2″-Aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (19A). Prepared from **18A** in 99% yield as described for **9Aa**: ^{1}H NMR δ 1.59 (s, 6H, C(CH_3)$_2$), 2.24 (s, 3H, ArCH_3), 2.55 (s, 3H, ArCH_3), 2.86 (s, 2H, CH_2COOH), 3.09 (s, 2H, NHCH_2), 3.68 (s, 2H, CH_2O), 3.78 (s, 2H, OCH_2), 4.41 (s, 2H, CH_2OC(=O)O), 5.85 (br s, 1H, NH_2), 6.67 (s, 1H, ArH), 6.85 (s, 1H, ArH); ^{13}C NMR δ 20.05, 24.93, 31.51, 38.77, 39.61, 47.43, 66.18, 66.87, 69.10, 122.44, 132.57, 133.47, 136.22, 138.21, 149.71, 154.22, 169.75, 175.20.

3-(2′-2″-(2″-PEG-aminoethoxy)ethoxycarbonyloxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (20A). Prepared from **19A** in 92% yield as described for **10Aa**: ^{13}C NMR δ 19.35, 24.36, 30.54, 37.66, 37.98, 46.40, 65.67, 67.68–70.61 (PEG), 121.55, 131.53, 133.01, 134.99, 137.17, 149.06, 152.91, 169.23, 171.84.

PEG-carbonate-TML–DNR (21A). Prepared from **20A** in 90% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.4 wt %: ^{13}C NMR δ 16.04, 19.28, 23.89, 24.59, 28.38, 30.93, 32.32, 34.23, 37.63, 38.91, 44.47, 55.82, 66.32, 66.84, 67.71–71.74 (PEG), 75.66, 78.33, 100.06, 110.20, 110.36, 117.90, 118.77, 119.81, 121.68, 131.85, 132.82, 133.46, 134.44, 135.00, 135.64, 137.49, 149.20, 153.46, 154.84, 155.52, 160.15, 169.38, 185.62, 185.91, 210.92.

1-*O*-(*tert*-Butyldimethylsilyl)-3-[2′-(2″-amino-2-Boc-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropanol (22A). Triphosgene (308.2 mg, 1.04 mmol) and pyridine (307.1 mg, 3.88 mmol) were added to a solution of **4A** (500 mg, 1.55 mmol) in chloroform (30 mL) and the mixture stirred at 35–40 °C for 3 h followed by cooling to room temperature. 2-Amino-2-[(*tert*-butoxycarbonyl)amino]ethylene glycol diethyl ether[24] (967.7 mg, 3.88 mmol) and pyridine (307.1 mg, 3.88 mmol) were added to the reaction solution and the mixture stirred at 35–40 °C overnight. The reaction solution was washed with 0.5 N HCl (3 × 10 mL) and water (10 mL) and dried over anhydrous $MgSO_4$, followed by removal of the solvent in vacuo. The residue was purified by silica gel column chromatography (30–40% EtOAc in hexane) to give product (720 mg, 78%): ^{1}H NMR δ −0.01 (s, 6H, 2 × Si(CH_3)$_2$), 0.86 (s, 9H, SiC(CH_3)$_3$), 1.45 (s, 9H, OC(CH_3)$_3$), 1.49 (s, 6H, 2 × C(CH_3)$_2$), 2.05 (t, J = 7.43, 2H, CH_2CH$_2$OSi), 2.23 (s, 3H, ArCH_3), 2.52 (s, 3H, ArCH_3), 3.34 (m, 2H, CH_2NHC(=O)O), 3.45–3.65 (m, 12H, 5 × CH_2 & CH$_2$$CH_2$OSi), 5.10 (br s, 1H, NH), 5.62 (bs, 1H, NH), 6.65 (s, 1H, ArH), 6.78 (s, 1H, ArH); ^{13}C NMR δ −5.40, 18.11, 20.08, 25.13, 25.84, 28.30, 31.68, 39.00, 40.24, 41.00, 46.04, 60.80, 70.14, 79.15, 123.20, 131.78, 134.36, 135.61, 137.89, 149.85, 155.03, 155.86; CI MS m/z 597 (M$^+$ + H, 80); HRMS calcd for $C_{31}H_{57}N_2O_7Si$ (M$^+$ + H) 597.3935, found 597.3933.

3-[2′-(2″-Amino-2-Boc-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropanol (23A). Prepared in 99% yield from **22A** as described for **6Ap**: ^{1}H NMR δ 1.37 (s, 9H, OC(CH_3)$_3$), 1.41 (s, 6H, 2 × C(CH_3)$_2$), 2.05 (t, J = 6.77, 2H, CH_2CH$_2$OH), 2.15 (s, 3H, ArCH_3), 2.43 (s, 3H, ArCH_3), 3.25 (m, 2H, CH_2NHC(=O)O), 3.35–3.56 (m, 13H, 5 × CH_2 & CH$_2$$CH_2$OSi & OH), 5.21 (bs, 1H, NH), 5.91 (bs, 1H, NH), 6.57 (s, 1H, ArH), 6.71 (s, 1H, ArH); ^{13}C NMR δ 20.04, 25.19, 28.27, 29.08, 31.80, 39.05, 40.21, 40.93, 45.92, 53.32, 60.15, 69.91, 70.11, 79.15, 123.22, 131.92, 134.28, 135.78, 137.98, 149.79, 155.54, 155.98.

3-[2′-(2″-Amino-2-Boc-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropanal (24A). Prepared from **23A** in 68% yield as described for **7Ap**: ^{1}H NMR δ 1.41 (s, 9H, OC(CH_3)$_3$), 1.54 (s, 6H, 2 × C(CH_3)$_2$), 2.20 (s, 3H, ArCH$_3$), 2.50 (s, 3H, ArCH_3), 2.77 (br s, 2H, CH_2CHO), 3.29–3.62 (m, 12H, 6 × CH_2), 5.07 (br s, 1H, NH), 5.66 (br s, 1H, NH), 6.64 (s, 1H, ArH), 6.79 (s, 1H, ArH), 9.53 (br s, 1H, CHO); ^{13}C NMR δ 21.12, 25.20, 28.30, 31.44, 38.05, 40.24, 41.05, 53.35, 56.71, 69.90, 70.12, 70.28, 79.18, 123.40, 132.20, 132.98, 136.54, 137.48, 149.44, 154.76, 155.90, 203.36.

3-[2′-(2″-Amino-2-Boc-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Acid (25A). Prepared from **24A** in 80% yield as described for **8Ap**: ^{1}H NMR δ 1.43 (s, 9H, OC(CH_3)$_3$), 1.58 (s, 6H, 2 × C(CH_3)$_2$), 2.22 (s, 3H, ArCH_3), 2.52 (s, 3H, ArCH_3), 2.79 (br s, 2H, CH_2COOH), 3.30–3.63 (m, 12H, 6 × CH_2), 5.28 (br s, 1H, NH), 6.02 (br s, 1H, NH), 6.68 (s, 1H, ArH), 6.78 (s, 1H, ArH); ^{13}C NMR δ 20.10, 25.08, 28.26, 31.15, 38.60, 40.09, 40.93, 47.92, 69.83, 70.11, 79.29, 122.93, 131.85, 133.85, 135.89, 137.52, 149.47, 155.05, 156.07, 175.09; CI MS m/z 497 (M$^+$ + H, 20); HRMS calcd for $C_{25}H_{41}N_2O_8$ (M$^+$ + H) 497.2863, found 497.2865.

3-[2′-(2″-Amino-2-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Acid (26A). Prepared from **25A** in 99% yield as described for **9Aa**: ^{1}H NMR δ 1.52 (s, 6H, 2 × C(CH_3)$_2$), 2.18 (s, 3H, ArCH_3), 2.50 (s, 3H, ArCH_3), 2.79 (br s, 2H, CH_2COOH), 2.91 (br s, 2H, CH_2NH$_2$), 3.36 (br s, 1H, NH), 3.56 (br s, 10H, 5 × CH_2), 6.32 (br s, 1H, NH), 6.59 (s, 1H, ArH), 6.77 (s, 1H, ArH); ^{13}C NMR δ 19.90, 24.99, 31.27, 31.67, 38.66, 39.55, 40.79, 47.98, 66.23, 69.89, 122.91, 131.94, 133.99, 135.89, 138.03, 149.41, 155.76, 160.90, 175.60.

3-[2′-(2″-PEG-amino-2-aminoethylene glycol diethyl ether carbamate)-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Acid (27A). Prepared from **26A** in 97% yield as described for **10Aa**: ^{13}C NMR δ 19.21, 24.27, 31.35, 37.53, 37.75, 39.98, 46.95, 69.51–70.27 (PEG), 122.04, 130.63, 133.35, 134.39, 136.53, 148.81, 154.08, 169.13, 172.13.

PEG-carbamate-TML–DNR (28A). Prepared from **27A** in 88% yield as described for **11Aa**. The amount of DNR present in this compound as measured by UV assay was 2.4 wt %: ^{13}C NMR δ 16.02, 19.21, 23.80, 24.67, 28.06, 31.28, 31.45, 32.25, 34.20, 37.59, 39.40, 40.22, 44.42, 48.61, 55.76, 66.23, 67.50, 67.76, 69.62–70.46 (PEG), 75.59, 100.00, 110.11, 110.28, 117.92, 118.70, 119.75, 122.30, 131.07, 132.77, 133.55, 134.37, 134.95, 135.52, 137.34, 149.61, 154.77, 155.17, 155.47, 160.10, 169.19, 169.76, 185.56, 185.82, 210.86.

8Aa–Ara-C (29Aa). A mixture of **8Aa** (700 mg, 1.78 mmol), ara-C (1.73 g, 7.12 mmol), HOBT (0.96 g, 7.12 mmol), and EDC·HCl (2.73 g, 14.25 mmol) in anhydrous pyridine (50 mL) was stirred at room temperature for 2 h followed by stirring at 40 °C overnight. The solvent was removed and DCM (50 mL) was used to dissolve the residue followed by water wash (3 × 30 mL) and 0.1 N HCl wash (2 × 30 mL). The organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed in vacuo to give the crude product which purified by silica gel column chromatography (5 to 10%, v/v, MeOH in DCM) to give 638.8 mg (52%) of product as a white solid: ^{1}H NMR δ 1.42, 1.55, 2.17, 2.26, 2.46, 2.79, 3.84, 3.91, 4.14, 4.33, 4.53, 5.49, 6.07, 6.17, 6.52, 6.76, 7.31, 7.67, 8.16, 8.62; ^{13}C NMR δ 17.77, 20.11, 25.36, 28.32, 31.51, 31.96, 39.57, 50.18, 50.45, 61.88, 74.50, 80.15, 85.90, 88.58, 96.25, 122.51, 132.82, 133.34, 136.73, 138.22, 146.57, 149.90, 155.65, 155.96, 162.08, 171.89, 174.06.

9Aa–Ara-C (30Aa). Prepared from **32Aa** in 82% yield as described for **9Aa**: ^{1}H NMR (DMSO-d_6) δ 1.52 (s, 3H, (CH_3)$_2$CH) 1.55 (s, 3H, (CH_3)$_2$CH), 1.62 (d, 1 H, J = 8.1, (CH$_3$)$_2$CH), 2.22 (s, 3H, ArCH_3), 2.57 (s, 3H, ArCH_3), 2.97 (s, 2H, CH_2C-

(=O)), 3.41–4.27 (m, 5 H, ara-C's H-2′-H-5′), 6.09 (d, 1H, J = 5.4, ara-C's H-1′), 6.67 (s, 1H, ArH), 6.90 (s, 1H, ArH), 7.12 (d, J = 5.4, ara-C's H-6), 8.05 (d, J = 8.1, ara-C's H-5), 8.67 (br s, 1H, TFA); ^{13}C NMR (DMSO-d_6) δ 15.45, 19.67, 24.97, 31.05, 31.23, 38.56, 40.41, 48.53, 49.02, 61.02, 64.94, 74.64, 76.14, 85.74, 86.95, 94.32, 122.32, 132.41, 134.08, 135.67, 138.09, 146.71, 149.20, 154.50, 158.21, 158.72, 162.02, 169.68, 171.87.

PEG-TML–Ara-C (31Aa). A solution of T-PEG (778 mg, 0.019 mmol), **30** (40 mg, 0.077 mmol), and DIEA (20 mg, 0.15 mmol) in anhydrous DCM (10 mL) was stirred at room temperature overnight. The solvent was removed in vacuo and the residue crystallized from 2-propanol to give 650 mg (84% yield) of the product **31Aa** as a white solid. The amount of ara-C present in this compound as measured by UV assay was 1.0 wt %: ^{13}C NMR δ 14.37, 18.58, 24.54, 31.30, 31.36, 38.66, 47.68, 49.09, 61.12, 67.54–71.05 (PEG), 74.76, 85.19, 86.54, 94.69, 121.25, 131.85, 133.08, 134.73, 137.35, 144.94, 148.58, 154.39, 160.88, 170.96, 171.26, 172.38.

Compound 32. Phenol was converted to carbamate **32** in 87% yield using the same procedures as described for **28A**: ^{13}C NMR δ 37.75, 40.22, 60.68, 67.40–69.40 (PEG), 78.28, 120.78, 124.24, 128.37, 148.80, 150.54, 169.15.

PEG-TML–Isopropylamine (33Aa). Prepared from **10Aa** and isopropylamine in 88% yield as described for **11Aa**: ^{13}C NMR δ 16.00, 19.50, 21.44, 21.54, 25.00, 31.32, 31.60, 39.33, 39.83, 47.94, 48.10, 67.48–71.55 (PEG), 77.92, 120.80, 131.85, 132.0, 133.6, 135.7, 138.2, 149.5, 169.34, 169.91, 171.56.

2. Biological Results. Cell Lines and Cytotoxicity Assays. Studies using P388/0 cell lines for IC_{50} (drug concentration inhibiting growth of cells by 50%) were maintained and conducted as previously reported.[22] Briefly, for IC_{50} determination, cells were seeded into the microwell plates at a density of 2×10^3 cells/50 μL/well. Plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 3 days. Cell growth was measured by the addition of 10 μL/well of Alamar Blue (Alamar Biosciences, Inc., Sacramento, CA) and the plates were incubated a further 4 h at 37 °C. The IC_{50} values for each compound were determined from absorbance vs dilution factor plots.

All cell cultures for animal implantation were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2 and subcultured once a week. All cell lines were periodically tested for Mycoplasma and were Mycoplasma free. M109 (Madison 109 murine lung carcinoma, National Cancer Institute (NCI), Bethesda, MD) was adapted to monolayer culture and grown in EMEM (Eagle's modified essential medium) with 10% fetal bovine serum (FBS). SKOV3 (human, ovarian adenocarcinoma, ATCC/HTB77) was raised in McCoy's 5a medium supplemented with 15% FBS. PC-3 (human, prostate adenocarcinoma, ATCC/CRL1435) was maintained in Ham's F12K medium with 7% FBS. MX-1 (human, mammary carcinoma) was obtained from the NCI and maintained in serial subcutaneous passages in nude mice.

In Vivo Antitumor Activity and PEG Linker Toxicity. Primary screening for antitumor activity against M109 tumors was conducted in 20–25 g, female *balb/c* mice (Hilltop Labs, Scottdale, PA). Mice were implanted (day 0) subcutaneously at the left flank with approximately 1×10^6 cells in 100 mL. Intraperitoneal treatments were administered on days 1 and 4, while intravenous dosages were given on days 3 and 6. Treatment groups (6/group) consisted of untreated controls, DNR, and seven PEG–DNR derivatives. For solid tumor xenograft studies, female *nu/nu* mice (Harlan Sprague–Dawley, Madison, WI), 18–24 g, were inoculated subcutaneously at the left flank with either a fragment of donor tumor tissue (MX-1) or a tumor cell suspension (1×10^6 cells) in 0.1 mL of PBS (SKOV3 and PC-3). Treatments began when the mean tumor volume reached approximately 75 mm^3 (day 1). The xenograft panel compared the efficacy of free DNR and PEG-TML–DNR (**11Aa**) administered intravenously at 3 mg/kg/dose (6/group) on days 1, 5, and 9 with control (untreated). Antitumor activity for all studies was calculated and determined as previously described.[17] To determine the toxicity of **33Aa**, ICR mice (Harlan Sprague–Dawley, Madison, WI) were intravenously treated (25 mg/mouse) with this simple PEG linker conjugate. Mice (4/group) were observed daily and weighed twice weekly for 2 weeks. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society of Medical Research and the *Guide for the Care and Use of Laboratory Animals* published by the NIH. These experimental protocols were approved by the Institutional Animal Care and Use Committee of UMDNJ–Robert Wood Johnson Medical School.

Statistics. The differences between treatment groups were assessed by one-way ANOVA. Multiple comparisons, when significant differences existed, were determined by least significant differences techniques. Statistical analysis was conducted using the StatView software program (Abacus Concepts, Inc., Berkeley, CA).

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